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THE DETERMINATION OF TRISATURATED TRIGLYCERIDES

by

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FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled

THE DETERMINATION OF TRISATURATED TRIGLYCERIDES

submitted by Eduard W. Kerkhoven in partial fulfilment of the requirements for the degree of Master of Science in Food Chemistry.

ABSTRACT

Trisaturated triglycerides (GS_3) were determined by a method based on the addition of mercuric acetate to the double bonds of the unsaturated triglycerides (GU). The reacted GU fraction was absorbed on a Florisil column, and the GS_3 fraction eluted unchanged and quantitatively. The reacted unsaturated glycerides could be eluted during a second elution with simultaneous regeneration into their original chemical configurations.

The average GS_3 content of 19 butterfat samples was found to be 39.7% by weight. Only minor differences existed between the GS_3 contents of three butterfat samples before and after randomization. The GS_3 contents of various fats other than butterfat were also determined.

The fatty acid compositions of the GS_3 and GU fractions of the three butterfat samples before and after randomization were found to be similar. The fatty acid compositions of GS_3 fractions of 19 butterfat samples indicated that the distribution of the fatty acids among the butterfat triglycerides appeared to have a random pattern. This appearance was lost when butterfat was fractionated by fractional crystallization from acetone. Butyric acid seemed to be combined with palmitic acid in the high melting glycerides and with oleic acid in the low melting glycerides.

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INTRODUCTION

Trisaturated glycerides (GS_3) are those glycerides which do not possess any double bonds in any of the fatty acid residues. The other glyceride classes are:

> Disaturated triglycerides (GSUS and GSSU), containing one or more sites of unsaturation in one fatty acid residue.
>
> Monosaturated triglycerides (GUSU and GUUS), containing one or more sites of unsaturation in two fatty acid residues.
>
> Triunsaturated triglycerides (GU_3), containing one or more sites of unsaturation in all three fatty acid residues.

GS_3 contents are determined to obtain a better understanding of the triglyceride structure in natural fats. Many theories of glyceride structure are based on the GS_3 contents of fats and oils.

Several techniques have been used for the determination of the GS_3 content. With other than the fats of simple fatty acid composition these have not proven very reliable. The reason for this difficulty is the peculiar physical behavior of the short chain saturated fatty acids. With some methods the GS_3 content is not actually determined but calculated from other data with the aid of assumptions regarding glyceride structure which may or may not be fully justified.

In the method outlined in this thesis, use has been made of the ability of the double bonds in unsaturated glycerides to react with mercuric acetate. The unsaturated triglycerides (GU) were reacted with mercuric acetate in methanol during a 30 minute reflux period and absorbed on a Florisil column as their mercuric acetate addition compounds. The GS_3 or unreacted part of the triglycerides is eluted unchanged and quantitatively from the column and weighed.

REVIEW OF LITERATURE

A. METHODS FOR THE DETERMINATION OF TRISATURATED GLYCERIDES CONTENT

(a) Oxidation methods

Hilditch and Lea (30) were the first to determine the GS_3 contents of fats and oils by oxidation of the double bonds of the fatty acids of the glycerides with powdered potassium permanganate in acetone solution. The double bonds undergo fission the formation of a carboxylic acid group, the oleo-, linoleo-, linoleno-, and eleostearoglycerides being converted to the corresponding azelaoglycerides. The unattacked trisaturated glycerides markedly differ in properties from the glycerides resulting from the oxidation. The two fractions are separated by washing with a mildly alkaline solution. Hilditch pointed out the difficulties encountered with emulsions that form during the separation of the neutral and acidic triglycerides. He used this technique to furnish evidence for his theory of even distribution of fatty acids in natural fats (13).

Kartha (38) claimed that considerable hydrolysis of the ester groups of a fat occurred during the potassium permanganate oxidation. He recommended that the oxidation be carried out in the presence of a slight excess of acetic acid to neutralize the alkaline materials that are formed. The restricted random theory, introduced by Kartha, is based on the results obtained by this technique (39).

Eshelman and Hammond (23) confirmed that ester hydrolysis may occur. They showed that if the oxidation was carried out in the presence of acetic acid, synthesis of new ester groups occurred and a neutral side product was formed. It was believed that the product was an acetic acid ester of a ketol, formed by partial oxidation of the double bonds.

Hammond and Eshelman (26) showed that both the methods of Hilditch and Kartha for oxidizing glycerides with potassium permanganate were unreliable. Hilditch's method was found to cause significant hydrolysis of the ester groups, while Kartha's method was shown to introduce new ester groups. Attempts to prevent saponification accompanying the oxidation by using agents other than acetic acid were unsuccessful. Hilditch (29) pointed out that the hydrolysis of the mono-azelaoglycerides is most serious, not during the oxidation, but during the subsequent separation of the alkaline washing.

(b) Fractional crystallization methods

Fractional crystallization is a second method introduced by Hilditch (29). It depends on the difference in solubility of long chain saturated and unsaturated fatty acids of the triglycerides. The method is only reliable for very simple fats. It does not permit complete separation of the fat into individual triglycerides. The most widely used solvent is acetone at a concentration of 10% w/v. The acetone used should be anhydrous, since traces of water have a marked effect on glyceride solubilities. Starting at a temperature of -60 or -70 C for the most unsaturated oils, the glycerides to be separated are crystallized at progressively higher temperatures.

The advantage of fractional crystallization is that all of the fractions are retained for further analysis. It is assumed that not more than two classes of glycerides are present in any fraction. However, there is evidence that this assumption may not be justified where several unsaturated fatty acids or short chain fatty acids are present.

The method requires large amounts of sample and large volumes of solvent (12, 31).

A similar method is the isotope dilution method of Calvin (9) using labeled tripalmitin. Trisaturated glycerides were isolated from the fats by crystallization from acetone. It was assumed that the trisaturated glycerides behaved as a single compound, whose solubility was the same as that of tripalmitin. However, trisaturated glycerides completely free from unsaturated glycerides could not be obtained. A correction had to be applied in the calculation of the GS_3 content (55).

(c) Countercurrent distribution

Countercurrent distribution is the fractionation of a mixture of substances by repeated partition or distribution between two immiscible liquid phases. With completely automatic equipment containing hundreds of many tubes separations can be made that would be practically impossible if the distributions were performed individually in separatory funnels.

Although the resolution is higher than fractional crystallization, accurate results for the determination of trisaturated glycerides could not be obtained (21, 22, 57, 58, 59, 60).

(d) Lipase hydrolysis

In 1960 Vander Wal (62) described the 1,3-random-2-random distribution hypothesis. He based this hypothesis on a procedure for calculating the molecular composition of natural fats, and the trisaturated glycerides were one of the six glyceride classes. In 1964 he rephrased this hypothesis (63). The hypothesis was based on the findings of Mattson and Beck (49), and Mattson and Lutton (50). They

showed that the fatty acids in the 1- and 3- positions of the triglycerides may be preferentially and non-specifically removed with little displacement of those in the 2- positions. The calculation of the six classes of triglycerides are derived from the data of:

(i) the percentage of saturated fatty acids in the whole fat, and

(ii) the percentage of saturated fatty acids in the 2-monoglycerides which would be produced if all the fatty acids in the 1- and 3- positions were removed by hydrolysis.

There are four possible sources of error:

(i) lack of specificity of the enzyme (11, 12).

(ii) evidence of a preferential attack of pancreatic lipase on butterfat glycerides containing shortchain fatty acids (8).

(iii) the formation of 1,3- diglycerides from 1,2- diglycerides due to isomerization (53).

(iv) The production of glycerol during hydrolysis, so that some of the free fatty acids must have come from the 2- positions of the original triglycerides (12).

(e) Chromatographic methods

1. Separation with silver nitrate impregnated silica gel

Column chromatography

Winstein and Lucas (66) reported in 1938 that the reaction between Ag^+ and alkenes is reversible and that the equilibrium is reached very readily. The chromatographic separations on silica gel impregnated with silver nitrate depend on the ability of compounds having a double bond to form co-ordination complexes with silver

ions (1). Compounds with trans double bonds have higher Rf values than those with the same number of double bonds but in the cis configuration. The chain length has no influence on the separation (42).

De Vries (17, 18) separated methyl oleate from methyl elaidate, methyl esters according on their degree of unsaturation, and positional isomers of triglycerides. He separated also closely related synthetic- and palm oil triglycerides (19). These separations by column chromatography are a promising method, because the triglycerides are not altered, but remain in their original chemical configuration. In contrast to separations by means of mercuric acetate no chemical operations preceeding and following the chromatographic procedure have to be carried out. It has not been investigated whether complexed butterfat triglycerides can be separated according to their degree of unsaturation.

Thin layer chromatography

The most recent and most promising method by which the glyceride classes of oils and fats are fractionated is by thin layer chromatography on silica gel impregnated with silver nitrate.

Kaufmann and Wessels (42) separated glycerides on their degree of unsaturation with silica gel impregnated with silver-nitrate, and followed this by a reversed phase separation according to the chain length of the glycerides. The triglyceride structure of each class could be identified by using pancreatic lipase combined with gas liquid chromatography. Beet oil having six different fatty acids was separated into 56 fractions which coincides with the theoretical maximum of 56 different triglycerides, not including positional or optical isomers.

Recently similar work was performed by Kuksis and McCarthy (44), Subbaram and Youngs (61), Litchfield et al. (47), Kaufmann et al. (40), Blank et al. (6), and Juriens and Kroesen (37).

2. DEAE - cellulose chromatography

Eshelman, et al. (24) have described a method based on the reaction of mercaptoacetic acid with unsaturated fatty acids of glycerides with subsequent column chromatography on DEAE - cellulose. The reacted glycerides are absorbed and the neutral saturated portion passed through the column. This residue had to be recycled two, three or four times, depending on the type of fat. A correction value had to be used, since the saturated triglycerides were still contaminated with unreacted unsaturated glycerides. This method was used for butterfat, but the unsaturated portion was lost for further investigation.

(f) Combined methods

1. Oxidation and hydrolysis

Youngs (69) described a combination of the techniques of oxidation and pancreatic lipase lipolysis as a means of determining the six glyceride classes of fats. The unsaturated fatty acids of the whole fat were oxidized to the corresponding dicarboxylic acids, followed by a liquid - liquid partion column chromatography into two fractions. One contained glycerides having no dicarboxylic acids or one dicarboxylic acid, and the second contained two or three dicarboxylic acids. Analysis of these fractions by gas liquid chromatography allowed the calculation of the proportions of the six triglyceride classes.

2. Ozonolysis and reduction

Another combination method (52) is the ozonolysis of a small sample in methylene chloride at -70'C, followed by reduction with a Lindlar (lead poisened platimum) catalyst with the formation of aldehydes. The mixture is then subjected to thin layer chromatography. The spots are visualized by charring after spraying with 50% sulfuric acid, and can then be measured with a densitometer. The four main classes of triglycerides are analyzed by this method.

B. <u>THE ADDITION OF MERCURIC ACETATE TO THE DOUBLE BONDS</u>

Hofmann and Sand (32), were the first to investigate the reactions of the mercuric salts with ethylenic compounds. Their work did not include the addition of mercuric acetate to fatty acids, their methyl esters, or triglycerides. In 1900 they reported that the reaction of mercuric nitrate with ethylene is as follows:

$$C_2H_4 + Hg(NO_3)_2 \longrightarrow NO_3Hg\text{-}C_2H_4\text{-}NO_3$$

In the fat field Leys (46) was the first to report the reaction of mercuric acetate with oleic acid in 1907.

In 1939 Ralston (54) reported that the reaction of mercuric acetate with oleic acid in the presence of methanol is as follows:

$$R\text{-}CH{=}CH\text{-}R \quad \xrightarrow[\substack{CH_3OH \\ \text{reflux 3 hrs.}}]{Hg(OAc)2} \quad \begin{array}{l} R\text{-}\underset{\substack{| \\ Hg \\ | \\ O \\ | \\ Ac}}{CH}\text{-}\underset{\substack{| \\ O \\ | \\ CH_3}}{CH}\text{-}R \end{array} \quad + \quad HOAc$$

He described the use of the adduct as a weed killer.

In 1927 Bertram (4) was the first to use the mercuric acetate addition reaction to separate saturated from unsaturated fatty acids, in order to obtain 99.5 - 99.6% pure oleic acid.

The purity of the oleic acid was measured by his oxidation method (2, 3). This method is similar to Hilditch's oxidation method for triglycerides, but was published earlier.

Bertram regenerated the adducts into the original compounds with hydrochloric acid. In 1958 Jantzen and Andreas (34) reported that the regeneration could be done with a 5% HCl solution. They also reported that it was possible to refractionate the reacted fatty acids according to their degree of unsaturation on silica gel.

Birks and Wright (5) described the use of peroxides and borontrifluoride etherate as catalysts in the reaction with stilbene. They, and Brandt and Plum (7) showed that the reaction is a second order reaction. Jantzen and Andreas (35) reported that the addition is a second order reaction, or perhaps, because of the exess of methanol, is close to a first order reaction. Unsaturated <u>cis</u> fatty acids reacted more readily than the <u>trans</u> isomers, by this means the <u>cis</u> isomers could be separated from the <u>trans</u> isomers (35, 36).

Unsaturated fatty acids or their methyl esters were separated from saturated fatty acids by filtration (4), on a Florisil (68), alumina (64), or silica gel (25) column, by liquid - liquid extraction (56, 65), paper chromatography (33), and by counter current distribution (35).

EXPERIMENTAL PROCEDURE

(a) Preparation of butterfat and margarine fat and randomization (interesterification)

The butterfats were obtained from butter by melting, washing with water to remove non-lipid material, drying under reduced pressure, and filtration. Randomization (interesterification) was carried out with 0.2% sodium methoxide catalyst at 50°C as described by de Man (14). Lard, shortening and coconut oil were commercial products used without further purification.

(b) Reaction with mercuric acetate in methanol

700 - 800 mg of sample were weighed into a 50 ml Pyrex ground joint Erlenmyer flask, and 3.0 g of mercuric acetate, 12 ml methanol and some boiling stones added. After the addition of methanol 2-3 drops of perchloric acid were added. The mixture was refluxed with a water cooled condenser on a hot plate for 30 minutes.

(c) Washing of the reaction mixture

The refluxed material was poured directly into a 500 ml Kimax saporatory funnel with a Teflon stopcock, containing 200 ml distilled water. The 50 ml Pyrex Erlenmyer flask was rinsed 3-5 times with a total volume of 75 ml chloroform. The organic bottom layer was removed when the layers were clearly separated. The water layer was then washed at least four times with 60 ml portions of chloroform. The combined chloroform extracts were allowed to evaporate at room temperature, with the aid of a fan. The last chloroform was evaporated in a vacuum oven at room temperature.

(d) Column chromatography on Florisil

Florisil (magnesium silicate) was dehydrated for 18 hours at 65°C in a vacuum oven. At least one day prior to use the Florisil was deactivated with 14% w/w distilled water. The columns were Pyrex tubes with a Teflon stop, about 40 cm long and 2 cm in diameter. The eluent was received in a weighed Erlenmeyer flask. The solvent was evaporated first on the steam bath and finally in a 65°C vacuum oven for one hour. The eluent solvent was 500 ml hexane - diethyl ether (8:2 v/v). The column was packed with part of the eluent solvent and 30 g of deactivated Florisil.

The reacted fat was dissolved with approximately 10 ml solvent and poured into the column. The flow rate was approximately 200 ml of eluent per hour. The container was rinsed with five 10 ml portions of solvent, the column was then filled to the top and the rest of the solvent added at regular intervals.

(e) Preparation of the saturated fraction for fatty acid composition determination by gas liquid chromatography.

The trisaturated glycerides were transferred with petroleum ether (boiling range 37 - 59°C) into a 100 ml beaker and placed on a steam bath. When about 30 ml solvent were left and still warm a spatula tip of activated corbon (Atlas Chemical Industries, Inc. "Darco" grade KB) and 0.5 g anhydrous Na_2SO_4 were added and the mixture filtered through a #42 Whatman filter paper.

The petroleum ether was evaporated from the filtered solution until about 10 ml were left, and transferred to a freeze drying bulb. The remainder of the petroleum ether was evaporated on the steambath, and dried at the water pump in a 60°C water bath.

(f) Recovery of the unsaturated fraction and its preparation for fatty acid determination by gas liquid chromatography

The reacted unsaturated fraction which is absorbed on the Florisil, was released by the addition of a second elution solvent consisting of 180 ml ethanol (95%), chloroform, HCl (9:8:1 v/v/v). The HCl will regenerate the adducts to their original unsaturated configuration (34).

The eluent was received in a 600 ml beaker filled with 200 ml distilled water and 200 ml petroleum ether. This mixture was transferred to a 500 ml Kimax saporatory funnel with a Teflon stopcock, and washed at least six times with 200 ml portions of distilled water.

The organic layer was evaporated on a steam bath until about 60 ml were left. Two drops of phenolphthalein and sufficient 0.1 N KOH in methanol were added to neutralize the solvent to the phenolphthalein end point. Further treatment was identical to that of the saturated fraction.

(g) Determination of the fatty acid composition

The fatty acid composition of the original fat, and the saturated and unsaturated fractions were determined by the method of de Man (16). Two parts by volume of 0.025 N potassium methoxide in

anhydrous methanol were added to one part by weight of fat in the freeze drying bulb. The unsaturated fraction should not become pinkish due to the presence of phenolphthalein if it is filtered properly. The bulb was sealed and if the fat was free from impurities, e.g. moisture, the methyl esters were formed within 30 minutes in a 60°C oven. The transesterification has come to completion if the two initial layers change into one.

After transesterification the bulb was opened and the methanol-methyl ester mixture injected directly into the column of an F & M, model 720, dual column programmed temperature gas chromatograph. The columns were 10 ft., 1/4 in. stainless steel, packed with 20% w/w stabilized diethylene glycol succinate, (Wilkens Instrument & Research Inc. 07-141), on 60/80 mesh Firebrick. Results are expressed as weight percent of methyl ester.

(h) Fractional crystallization from acetone

Crystals were filtered at successively lower temperatures starting at 15°C at 10°C intervals until -45°C. In this way seven fractions and a residue were obtained. This method will be described in more detail by Pi-chen Chen (51). These products were analyzed by the procedure outlined above.

RESULTS

Table 1 lists the GS_3 contents of non randomized and randomized samples of June, September and December butterfat. There were only minor differences between the GS_3 contents of non randomized and randomized butterfat. Table 3 gives the GS_3 contents of July butterfat and its fractions obtained by fractional crystallization from acetone. The high melting glycerides have higher GS_3 contents than the low melting glycerides, although the +5°C fraction has a slightly higher GS_3 content than the +15°C fraction. Table 2 lists the GS_3 contents of 19 butterfat samples. These butterfats were obtained from butter samples produced by local creameries. The GS_3 contents range from 33.2% to 44.5%, the average being 39.7% with a standard deviation of 1.34. Table 4 outlines the GS_3 contents of some fats other than butterfat.

Tables 5, 6 and 7 show the fatty acid composition of June, September and December butterfat respectively, and the fatty acid compositions of the GS_3 and GU fractions of these samples before and after randomization. The fatty acid compositions of the GS_3 and GU fractions before and after randomization appear to be very similar, suggesting a random pattern of fatty acid distribution among the triglycerides in the whole fat.

Table 8 shows the fatty acid composition of the 19 butterfat samples. With the possible exception of sample 9, these fatty acid compositions also suggest a random pattern of the fatty acid distribution among the triglycerides of butterfat. Table 9 gives the fatty acid composition of two lard samples, the GS_3 of these two samples, and the GU of sample 1. These samples were purchased from local stores.

Table 10 gives the fatty acid composition of two shortening samples, the GS_3 of these two samples, and the GU of sample 1. Table 11 outlines the fatty acid compositions of five margarine fat samples, and the GS_3 fractions of these samples. The samples were purified from margarine samples purchased in local stores. The margarines from which 1,2 and 4 were obtained, were stated to contain only vegetable oils, samples 3 and 5 were stated to contain vegetable and marine oils. Table 12 lists the fatty acid compositions of coconut oil, and the GS_3 and GU fraction of this oil.

Table 13 outlines the fatty acid compositions of July butterfat, the fractions obtained from fractional crystallization from acetone, and the GS_3 portions of these fractions. From these fatty acid compositions it can be concluded that butyric acid is combined with palmitic acid in the high melting glyceride fractions and with oleic acid in the low melting glyceride fractions.

Table 14 outlines the glyceride types of June, September, and December butterfat samples before and after randomization. The contents are expressed in mole percent. The GS_3 contents are as determined by the mercuric acetate method. The other glyceride types were found by calculation using the method of Hammond and Jones (28) for the non randomized samples. The randomized butterfat samples were calculated according to the theory of random distribution.

Figure 1 shows gas chromatograms of the fatty acid compositions of June whole butterfat, and the GS_3 and GU fractions of this butterfat. Figure 2 shows the chromatograms of the fatty acid compositions of three fractions obtained by fractional crystallization from acetone of

July butterfat. The three fractions are the +15°C fraction which is a high melting glyceride fraction, the -15°C fraction which closely resembles the whole fat, and the residual fraction which is a low melting glyceride fraction. Figure 3 shows the chromatograms of the fatty acid compositions of the saturated triglycerides of these fractions obtained by fractional crystallization from acetone.

Table 1. GS_3 content of butterfat and randomized butterfat

Product		GS_3 % $^w/_w$
June	non randomized	38.0
June	randomized	38.3
September	non randomized	35.9
September	randomized	35.8
December	non randomized	37.8
December	randomized	37.6

Table 2. GS_3 content of 19 butterfat samples

Sample number	GS_3 % $^w/w$
1	37.9
2	41.6
3	38.8
4	37.7
5	37.7
6	33.2
7	41.3
8	35.2
9	36.1
10	43.6
11	41.5
12	44.3
13	43.1
14	37.9
15	43.8
16	42.6
17	41.4
18	38.4
19	38.5

Table 3. GS_3 content of July butterfat and its fractions obtained by fractional crystallization from acetone

Product		GS_3 % $^w/w$
July	butterfat	38.4
+15°C	fraction	67.2
+ 5°C	fraction	68.6
- 5°C	fraction	52.3
-15°C	fraction	43.7
-25°C	fraction	32.2
-35°C	fraction	30.6
-45°C	fraction	27.5
residue	fraction	27.2

Table 4. GS_3 content of some fats and some fat products

Product		GS_3 % $^{w}/w$
lard	1	11.1
lard	2	10.3
margarine fat	1	4.1
margarine fat	2	6.3
margarine fat	3	9.0
margarine fat	4	5.4
margarine fat	5	9.3
shortening	1	17.1
shortening	2	11.5
coconut oil		81.9

Table 5. Fatty acid composition (as weight % of methyl ester) of June butterfat and the trisaturated (GS_3) and unsaturated (GU) glycerides before and after randomization.

Fatty acid	Whole fat	GS_3 non randomized	GS_3 randomized	GU non randomized	GU randomized
4:0	3.5	4.9	5.1	2.7	2.4
6:0	2.1	2.9	2.9	1.8	1.8
8:0	1.3	1.7	1.7	0.9	0.9
10:0	2.8	4.0	4.1	2.1	2.0
10:1	0.2	0	0	0.3	0.3
12:0	3.2	4.8	5.0	2.2	2.4
14:0	10.4	16.0	15.6	7.8	8.3
A[1]	3.2	3.3	4.3	3.2	2.7
16:0	24.7	38.0	37.4	17.9	18.4
B[2]	3.2	2.8	2.6	3.2	3.6
18:0	13.4	19.8	19.6	10.1	10.5
18:1	25.7	trace	trace	38.7	38.6
18:2	3.4	0	0	5.4	5.3
18:3	1.6	0	0	2.6	2.5
a[3]	0	0.6	0.4	0.2	0.1
b[4]	0	0.3	0.2	0.2	0.3
d[5]	0.5	0.6	0.7	0.3	0.3

[1] Fatty acids with retention volumes between 14:0 and 16:0
[2] Fatty acids with retention volumes between 16:0 and 18:0
[3] Fatty acids with retention volumes between 4:0 and 6:0
[4] Fatty acid with retention volume between 8:0 and 10:0
[5] Fatty acids with retention volumes between 12:0 and 14:0

Table 6. Fatty acid composition (as weight % of methyl ester) of September butterfat and the trisaturated (GS_3) and unsaturated (GU) glycerides before and after randomization.

Fatty acid	Whole fat	GS_3 non randomized	GS_3 randomized	GU non randomized	GU randomized
4:0	3.6	5.2	5.8	3.0	2.6
6:0	1.9	3.0	2.9	1.4	1.3
8:0	1.0	1.6	1.6	0.7	0.7
10:0	2.1	3.4	3.3	1.5	1.4
10:1	0.2	0	0	0.3	0.3
12:0	2.6	4.1	4.0	2.0	2.0
14:0	9.9	14.8	15.2	7.5	7.2
A[1]	3.3	4.4	3.7	2.8	3.1
16:0	25.0	39.3	39.0	17.8	18.0
B[2]	3.1	2.8	2.5	3.2	3.4
18:0	13.3	19.5	19.9	10.2	10.0
18:1	27.5	trace	trace	40.1	40.4
18:2	3.7	0	0	5.5	5.4
18:3	2.4	0	0	3.4	3.4
a[3]	0	0.8	1.1	0.2	0.2
b[4]	0	0.3	0.2	0.1	0.1
d[5]	0.4	0.5	0.4	0.3	0.3

1 Fatty acids with retention volumes between 14:0 and 16:0
2 Fatty acids with retention volumes between 16:0 and 18:0
3 Fatty acids with retention volumes between 4:0 and 6:0
4 Fatty acid with retention volume between 8:0 and 10:0
5 Fatty acids with retention volumes between 12:0 and 14:0

Table 7. Fatty acid composition (as weight % of methyl ester) of December butterfat and the trisaturated (GS_3) and unsaturated (GU) glycerides before and after randomization.

Fatty acid	Whole fat	GS_3 non randomized	GS_3 randomized	GU non randomized	GU randomized
4:0	3.6	5.6	5.7	2.6	2.6
6:0	1.9	2.9	3.0	1.3	1.3
8:0	1.0	1.5	1.6	0.7	0.7
10:0	2.2	3.2	3.2	1.6	1.5
10:1	0.2	0	0	0.3	0.3
12:0	2.5	3.8	3.8	1.7	1.7
14:0	9.6	14.9	14.6	6.9	7.1
A[1]	3.3	3.5	3.5	3.1	3.1
16:0	27.2	41.3	41.1	20.3	20.5
B[2]	3.6	2.5	1.9	4.3	4.4
18:0	13.2	19.6	20.2	10.7	9.7
18:1	28.2	trace	trace	42.4	42.3
18:2	2.2	0	0	3.1	3.1
18:3	0.9	0	0	1.4	1.4
a[3]	0	0.6	0.6	trace	trace
b[4]	0	0.1	0.2	trace	trace
d[5]	0.4	0.5	0.5	0.3	0.3

1 Fatty acids with retention volumes between 14:0 and 16:0
2 Fatty acids with retention volumes between 16:0 and 18:0
3 Fatty acids with retention volumes between 4:0 and 6:0
4 Fatty acid with retention volume between 8:0 and 10:0
5 Fatty acids with retention volumes between 12:0 and 14:0

Table 8. Fatty acid composition (as weight % of methyl ester) of 19 samples of butterfat and the trisaturated glycerides of these butterfat samples

Fatty acid	Sample number 1 Whole fat	1 GS_3	2 Whole fat	2 GS_3	3 Whole fat	3 GS_3	4 Whole fat	4 GS_3
4:0	3.2	4.9	3.0	5.1	2.9	4.2	3.1	4.0
6:0	1.7	2.8	1.7	3.7	1.5	2.1	1.6	2.2
8:0	1.0	1.4	0.9	2.0	0.8	1.0	0.9	1.1
10:0	2.3	3.7	2.0	4.7	1.9	3.1	1.8	2.8
10:1	0.3	0	0.2	0	0.2	0	0.1	0
12:0	2.8	4.4	2.6	4.8	2.4	4.3	2.3	3.9
14:0	10.3	16.5	9.9	17.8	9.6	15.2	9.0	14.7
A[1]	3.0	3.3	3.2	3.8	3.3	4.5	2.4	3.5
16:0	24.2	42.3	29.0	40.3	28.3	42.1	26.6	43.0
B[2]	4.2	2.0	3.9	1.9	4.1	2.7	4.2	2.1
18:0	12.6	18.4	12.2	15.4	12.7	19.9	13.4	22.3
18:1	30.4	trace	28.0	trace	28.0	trace	30.4	trace
18:2	2.8	0	2.0	0	2.6	0	2.7	0
18:3	0.8	0	1.2	0	1.5	0	1.0	0
a[3]	0	0	0	0	0	0	0	0
b[4]	0	0	0	0	0	0	0	0
d[5]	0.3	0.3	0.2	0.3	0.2	0.4	0.2	0.4

[1] Fatty acids with retention volumes between 14:0 and 16:0
[2] Fatty acids with retention volumes between 16:0 and 18:0
[3] Fatty acids with retention volumes between 4:0 and 6:0
[4] Fatty acid with retention volume between 8:0 and 10:0
[5] Fatty acids with retention volumes between 12:0 and 14:0

Table 8. Fatty acid composition (as weight % of methyl ester) of 19 samples of butterfat and the trisaturated glycerides of these butterfat samples

	Sample number							
	5		6		7		8	
Fatty acid	Whole fat	GS_3	Whole fat	GS_3	Whole fat	GS_3	Whole fat	GS_3
4:0	3.3	4.8	3.2	5.0	2.4	4.0	3.1	5.5
6:0	1.8	2.4	1.7	2.5	1.5	2.1	1.8	2.6
8:0	1.0	1.4	1.0	1.4	0.8	1.4	1.1	1.6
10:0	2.3	3.3	2.2	3.4	1.9	2.8	2.6	3.8
10:1	0.3	0	0.2	0	0.2	0	0.3	0
12:0	2.7	4.3	2.6	4.3	2.5	3.7	3.3	5.0
14:0	10.2	16.4	9.4	15.8	9.4	15.0	9.9	16.1
A[1]	3.1	4.4	3.3	4.1	2.0	3.7	2.3	2.6
16:0	25.4	39.1	23.4	38.9	27.5	44.2	23.6	37.9
B[2]	3.1	1.6	4.0	3.2	2.7	2.6	3.9	2.5
18:0	13.4	21.9	13.6	20.9	15.0	20.0	13.6	21.8
18:1	29.1	trace	28.5	trace	30.7	trace	29.3	trace
18:2	2.3	0	4.0	0	1.9	0	3.2	0
18:3	1.8	0	2.6	0	1.3	0	1.7	0
a[3]	0	0	0	0	0	0	0	0
b[4]	0	0	0	0.2	0	0	0	0
d[5]	0.2	0.3	0.2	0.3	0.2	0.4	0.3	0.3

[1] Fatty acids with retention volumes between 14:0 and 16:0
[2] Fatty acids with retention volumes between 16:0 and 18:0
[3] Fatty acids with retention volumes between 4:0 and 6:0
[4] Fatty acid with retention volume between 8:0 and 10:0
[5] Fatty acids with retention volumes between 12:0 and 14:0

Table 8. Fatty acid composition (as weight % of methyl ester) of 19 samples of butterfat and the trisaturated glycerides of these butterfat samples

	Sample number							
	9		10		11		12	
Fatty acid	Whole fat	GS_3	Whole fat	GS_3	Whole fat	GS_3	Whole fat	GS_3
4:0	4.5	5.1	3.6	5.0	4.2	5.3	2.2	4.2
6:0	2.8	2.5	1.9	3.0	2.0	2.9	1.4	2.0
8:0	1.6	1.6	1.2	1.9	1.2	1.6	0.8	1.2
10:0	3.2	3.9	2.5	4.3	2.4	3.3	2.0	2.8
10:1	0.3	0	0.2	0	0.1	0	0.1	0
12:0	3.6	4.9	3.0	5.1	2.7	4.2	2.6	3.7
14:0	10.3	17.3	10.2	16.4	8.6	14.7	9.8	14.4
A[1]	2.7	3.6	3.1	3.7	3.9	4.1	2.9	2.5
16:0	22.9	37.7	24.0	37.9	24.5	41.4	28.2	42.0
B[2]	2.3	2.6	5.0	2.3	6.1	3.8	3.6	2.4
18:0	12.8	20.5	13.1	20.1	11.0	18.9	12.5	24.6
18:1	28.5	trace	26.4	trace	27.9	trace	29.3	trace
18:2	2.2	0	3.1	0	3.2	0	2.5	0
18:3	2.1	0	2.5	0	2.0	0	2.0	0
a[3]	0	0	0	0	0	0	0	0
b[4]	0	0	0	0	0	0	0	0
d[5]	0.2	0.3	0.2	0.3	0.2	0.3	0.1	0.2

1 Fatty acids with retention volumes between 14:0 and 16:0
2 Fatty acids with retention volumes between 16:0 and 18:0
3 Fatty acids with retention volumes between 4:0 and 6:0
4 Fatty acid with retention volume between 8:0 and 10:0
5 Fatty acids with retention volumes between 12:0 and 14:0

Table 8. Fatty acid composition (as weight % of methyl ester) of 19 samples of butterfat and the trisaturated glycerides of these butterfat samples

	Sample number							
	13		14		15		16	
Fatty acid	Whole fat	GS_3	Whole fat	GS_3	Whole fat	GS_3	Whole fat	GS_3
4:0	3.8	4.4	3.5	5.2	3.5	4.5	3.8	4.8
6:0	1.8	2.4	1.8	2.8	2.0	2.6	2.0	2.8
8:0	0.9	1.4	1.0	1.7	1.0	1.3	1.1	1.5
10:0	1.8	3.0	1.9	3.5	2.2	3.1	2.5	3.6
10:1	0.1	0	0.1	0	0.2	0	0.2	0
12:0	2.2	3.5	2.3	4.2	2.8	4.2	2.8	4.5
14:0	9.0	13.7	9.0	15.1	9.6	12.3	9.3	14.9
A[1]	3.0	5.2	3.1	2.5	3.2	2.2	4.3	3.7
16:0	25.0	39.7	25.4	38.3	24.4	42.2	25.8	40.9
B[2]	2.8	2.0	3.9	3.0	2.1	1.6	4.3	3.3
18:0	14.5	24.1	14.5	23.4	13.1	24.2	12.3	19.6
18:1	31.7	trace	31.4	trace	32.2	trace	27.2	trace
18:2	2.0	0	1.7	0	2.2	0	3.6	0
18:3	0.3	0	0.3	0	1.1	0	0.6	0
a[3]	0	0.4	0	0	0	0.8	0	0.1
b[4]	0	0	0	0	0	0	0	0
d[5]	0.1	0.2	0.1	0.2	0.2	0.3	0.2	0.3

1 Fatty acids with retention volumes between 14:0 and 16:0
2 Fatty acids with retention volumes between 16:0 and 18:0
3 Fatty acids with retention volumes between 4:0 and 6:0
4 Fatty acid with retention volume between 8:0 and 10:0
5 Fatty acids with retention volumes between 12:0 and 14:0

Table 8. Fatty acid composition (as weight % of methyl ester) of 19 samples of butterfat and the trisaturated glycerides of these butterfat samples

	Sample number					
	17		18		19	
Fatty acid	Whole fat	GS_3	Whole fat	GS_3	Whole fat	GS_3
4:0	4.3	5.2	2.5	4.3	2.0	4.0
6:0	2.4	3.1	1.6	2.3	1.2	2.3
8:0	1.2	1.8	1.0	1.5	0.8	1.3
10:0	2.3	3.9	2.4	3.6	1.9	3.3
10:1	0.2	0	0.2	0	0.2	0
12:0	2.6	4.6	2.9	4.6	2.5	4.1
14:0	9.2	15.5	10.1	15.8	10.8	16.9
A[1]	2.7	2.6	2.4	5.2	2.1	4.0
16:0	25.8	39.9	23.4	36.1	26.0	40.8
B[2]	3.9	3.4	2.9	2.8	3.6	2.8
18:0	13.6	19.7	14.3	22.4	12.9	19.8
18:1	29.0	trace	29.5	trace	29.9	trace
18:2	2.2	0	3.9	0	4.1	0
18:3	0.5	0	2.8	0	2.2	0
a[3]	0	0	0	0.6	0	0.4
b[4]	0	0	0	0.2	0	0
d[5]	0.1	0.3	0.2	0.4	0	0.2

1 Fatty acids with retention volumes between 14:0 and 16:0
2 Fatty acids with retention volumes between 16:0 and 18:0
3 Fatty acids with retention volumes between 4:0 and 6:0
4 Fatty acid with retention volume between 8:0 and 10:0
5 Fatty acids with retention volumes between 12:0 and 14:0

Table 9. Fatty acid composition (as weight % of methyl ester) of two lard samples, the trisaturated glycerides (GS_3) of these samples and the unsaturated glycerides (GU) of sample 1

Fatty acid	Sample number 1			Sample number 2	
	Whole fat	GS_3	GU	Whole fat	GS_3
12:0	0	1.3	0	0	0.9
14:0	1.3	2.8	1.3	1.2	3.6
A[1]	0	0	0	0	0
16:0	24.7	45.4	23.7	25.1	49.1
B[2]	2.3	0.2	2.5	1.8	0
18:0	12.7	45.8	10.7	12.1	45.1
18:1	46.6	4.6	49.1	46.7	1.3
18:2	10.9	0	10.8	12.6	0
18:3	1.5	0	2.0	0.5	0

1 Fatty acids having retention volumes between 14:0 and 16:0
2 Fatty acids having retention volumes between 16:0 and 18:0

Table 10. Fatty acid composition (as weight % of methyl ester) of two shortening samples, the trisaturated glycerides (GS_3) of these samples, and the unsaturated glycerides (GU) of sample 1

	Sample number				
	1			2	
Fatty acid	Whole fat	GS_3	GU	Whole fat	GS_3
10:0	0	0.8	0	0	0
12:0	0	0.9	0	0	0
14:0	6.5	7.8	5.0	1.1	5.6
A[1]	0	1.8	0.2	0	0
16:0	26.3	47.4	20.9	14.5	38.9
B[2]	8.3	1.0	10.5	1.5	0
18:0	13.8	39.8	9.8	11.4	55.5
18:1	29.7	0.5	34.0	51.1	0
18:2	11.2	0	14.0	18.6	0
18:3	4.2	0	5.4	1.8	0

[1] Fatty acids having retention volumes between 14:0 and 16:0
[2] Fatty acids having retention volumes between 16:0 and 18:0

Table 11. Fatty acid composition (as weight % of methyl ester) of five margarine fat samples and the trisaturated glycerides (GS_3) of these samples

	Sample number									
	1		2		3		4		5	
Fatty acid	Whole fat	GS_3	Whole fat	GS_3	Whole fat	GS_3	Whole fat	GS_3	Whole fat	GS_3
8:0	0	0	0	0.2	0	0	0	0	0	
10:0	0	0	0	0.3	0	0	0	0	0	0.2
12:0	0	0	0.1	2.7	0	0	0	0	0.6	3.5
14:0	1.2	16.7	0.2	2.2	8.3	16.2	0.9	3.6	6.4	15.0
A[1]	0	0	0	0.7	0	0	1.3	0	0.3	0.8
16:0	12.3	60.7	13.4	53.6	23.4	59.8	9.4	37.6	21.2	53.0
B[2]	0	0	0	0	10.6	0	0	0	9.7	1.1
18:0	5.9	22.0	7.7	35.2	6.9	23.1	8.7	49.2	7.5	24.3
18:1	49.0	0.6	62.3	4.5	35.1	0.8	67.3	4.4	41.7	0.7
18:2	31.5	0	15.7	0.6	3.3	0	6.8	1.8	11.0	1.4
18:3	0	0	0.6	0	12.3	0	5.6	3.4	1.6	0

1 Fatty acids having retention volumes between 14:0 and 16:0
2 Fatty acids having retention volumes between 16:0 and 18:0

Table 12. Fatty acid composition (as weight % of methyl ester) of coconut oil and the tri-saturated (GS_3) and unsaturated (GU) glycerides of this sample.

Fatty acid	Whole fat	GS_3	GU
6:0	0.9	0.9	0.5
8:0	7.6	8.6	4.6
10:0	6.5	7.1	2.9
12:0	46.3	52.5	22.9
14:0	17.0	18.4	9.5
16:0	8.2	8.2	11.9
16:1	0.1	0	0.5
18:0	2.7	3.0	2.6
18:1	7.2	0.5	35.0
18:2	2.3	0.8	9.6
18:3	trace	0	trace

Table 13. Fatty acid composition of July fat, its fractions obtained by fractional crystallization from acetone, and the tri-saturated glycerides (GS_3) of July fat and its fractions

Fatty acid	July fat		+15°C Fraction[7]		+5°C Fraction[8]	
	Whole fat	GS_3	+15°C Fraction	GS_3	+5°C Fraction	GS_3
4:0	2.5	4.3	0	0	1.3	2.3
6:0	1.6	2.3	trace	trace	0.9	1.4
8:0	1.0	1.5	trace	trace	0.5	0.8
10:0	2.4	3.6	0.9	0.6	1.5	2.4
10:1	0.2	0	0	0	0	0
12:0	2.9	4.6	2.5	2.4	2.6	3.5
14:0	10.1	15.8	11.2	12.2	9.3	11.9
A[1]	2.4	5.2	2.1	1.3	2.6	3.6
16:0	23.4	36.1	33.8	41.0	32.9	39.0
B[2]	2.9	2.8	2.9	2.7	2.7	2.2
18:0	14.3	22.4	28.6	39.9	26.9	32.9
18:1	29.5	trace	14.4	0	16.2	0
18:2	3.9	0	3.3	0	2.0	0
18:3	2.8	0	0.3	0	0.6	0
a[3]	0	0.6	0	0	0	0
b[4]	0	0.2	0	0	0	0
c[5]	-	0	0	0	0	0
d[6]	0.2	0.4	0	0	0	0

[1] Fatty acids having retention volumes between 14:0 and 16:0
[2] Fatty acids having retention volumes between 16:0 and 18:0
[3] Fatty acids having retention volumes between 4:0 and 6:0
[4] Fatty acid having a retention volume between 8:0 and 10:0
[5] Fatty acid having a retention volume slightly smaller than 10:1
[6] Fatty acids having retention volumes between 10:0 and 12:0

[7] Yield 8.9% w/w
[8] Yield 9.8% w/w

Table 13. Fatty acid composition of July fat, its fractions obtained by fractional crystallization from acetone, and the tri-saturated glycerides (GS_3) of July fat and its fractions

Fatty acid	-5°C Fraction[9]		-15°C Fraction[10]		-25°C Fraction[11]	
	-5°C Fraction	GS_3	-15°C Fraction	GS_3	-25°C Fraction	GS_3
4:0	3.2	4.9	3.9	5.1	4.4	5.7
6:0	1.8	2.9	2.1	2.9	2.5	3.5
8:0	0.9	1.4	1.2	1.5	1.6	2.1
10:0	1.9	3.0	2.5	3.6	3.5	5.5
10:1	0.2	0	0.2	0	0.3	0
12:0	2.1	3.0	3.3	4.7	4.1	7.9
14:0	9.5	10.3	12.6	18.0	9.9	20.8
A[1]	2.3	2.8	2.5	3.9	3.1	5.1
16:0	29.8	41.6	23.9	39.3	16.9	31.5
B[2]	2.6	2.9	2.9	1.9	2.9	1.4
18:0	20.1	26.5	11.9	17.7	8.7	13.4
18:1	20.9	0	26.7	0	34.8	trace
18:2	3.5	0	4.5	0	4.4	0
18:3	1.2	0	1.4	0	2.6	0
a[3]	0	0	0	0.1	0	0.1
b[4]	0.1	0.7	0.2	1.1	0.2	2.2
c[5]	-	0	-	trace	-	0.2
d[6]	0	0	0	0.2	0	0.6

1 Fatty acids having retention volumes between 14:0 and 16:0
2 Fatty acids having retention volumes between 16:0 and 18:0
3 Fatty acids having retention volumes between 4:0 and 6:0
4 Fatty acid having a retention volume between 8:0 and 10:0
5 Fatty acid having a retention volume slightly smaller than 10:1
6 Fatty acids having retention volumes between 10:0 and 12:0

9 Yield 14.5% w/w
10 Yield 27.2% w/w
11 Yield 13.5% w/w

Table 13. Fatty acid composition of July fat, its fractions obtained by fractional crystallization from acetone, and the tri-saturated glycerides (GS_3) of July fat and its fractions

Fatty acid	-35°C Fraction[12]		-45°C Fraction[13]		Residue[14]	
	-35°C Fraction	GS_3	-35°C Fraction	GS_3	Residue	GS_3
4:0	4.3	5.8	4.9	6.5	7.3	7.1
6:0	2.6	4.2	2.9	4.6	3.6	5.3
8:0	1.7	2.9	1.8	3.5	2.2	4.5
10:0	3.5	7.6	3.6	7.8	3.5	9.1
10:1	0.4	0	0.4	0	0.5	0
12:0	3.6	9.4	3.6	9.0	3.1	8.0
14:0	7.8	16.9	8.3	17.0	7.5	16.0
A[1]	2.9	4.7	3.1	4.9	2.8	3.9
16:0	17.3	29.8	14.6	28.7	9.4	19.4
B[2]	3.8	2.7	4.2	2.8	3.7	2.2
18:0	6.7	12.0	6.0	12.6	2.0	6.5
18:1	36.8	trace	38.2	trace	44.5	trace
18:2	5.2	0	5.1	0	6.3	0
18:3	3.0	0	2.8	0	2.6	0
a[3]	trace	0.2	0.1	0.4	0.2	0.5
b[4]	0.2	3.0	0.3	1.6	0.6	16.8
c[5]	-	0.4	-	0.2	-	0.3
d[6]	0.2	0.5	0.2	0.4	0.2	0.4

1 Fatty acids having retention volumes between 14:0 and 16:0
2 Fatty acids having retention volumes between 16:0 and 18:0
3 Fatty acids having retention volumes between 4:0 and 6:0
4 Fatty acid having a retention volume between 8:0 and 10:0
5 Fatty acid having a retention volume slightly smaller than 10:1
6 Fatty acids having retention volumes between 10:0 and 12:0

12 Yield 9.1% w/w
13 Yield 5.0% w/w
14 Yield 13.1% w/w

Table 14. Glyceride types in butterfat before and after randomization. GS_3 content as determined by the mercuric acetate method. The non randomized glyceride types were found by calculation using the method of Hammond and Jones (28). The randomized glyceride types were calculated according to the theory of random distribution

Sample	Glyceride type, mole %			
	GS_3*	GS_2U	GSU_2	GU_3
June non randomized	38.8	39.7	18.7	2.8
June randomized	39.1	42.5	16.3	2.1
September non randomized	36.6	37.8	21.6	4.0
September randomized	36.5	41.9	18.8	2.8
December non randomized	38.6	38.2	19.9	3.3
December randomized	38.4	42.2	17.1	2.3

*The GS_3 contents of the randomized fats calculated according to the theory of random distribution were June 37.6%, September 32.9%, and December 35.9%.

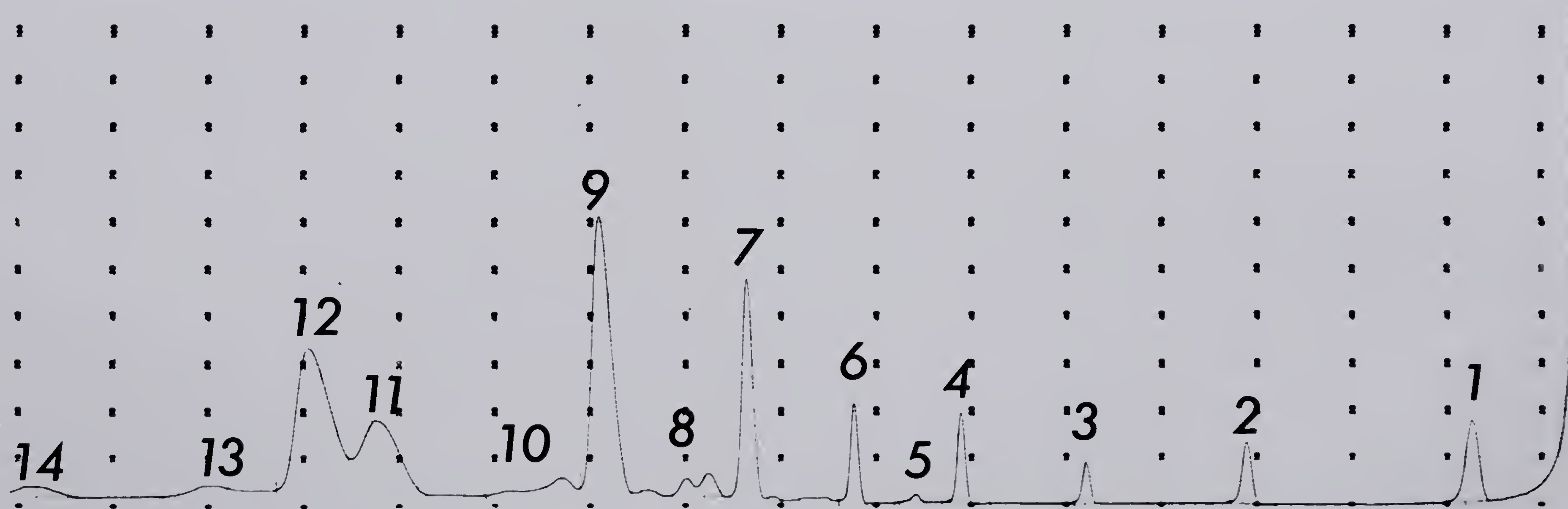

Fig. 1a. GAS CHROMATOGRAM OF METHYL ESTERS OF JUNE WHOLE BUTTERFAT.
Peaks in order of appearance: (1) 4:0, (2) 6:0, (3) 8:0, (4) 10:0, (5) 10:1, (6) 12:0, (7) 14:0, (8) group A, (9) 16:0, (10) group B, (11) 18:0, (12) 18:1, (13) 18:2, (14) 18:3.

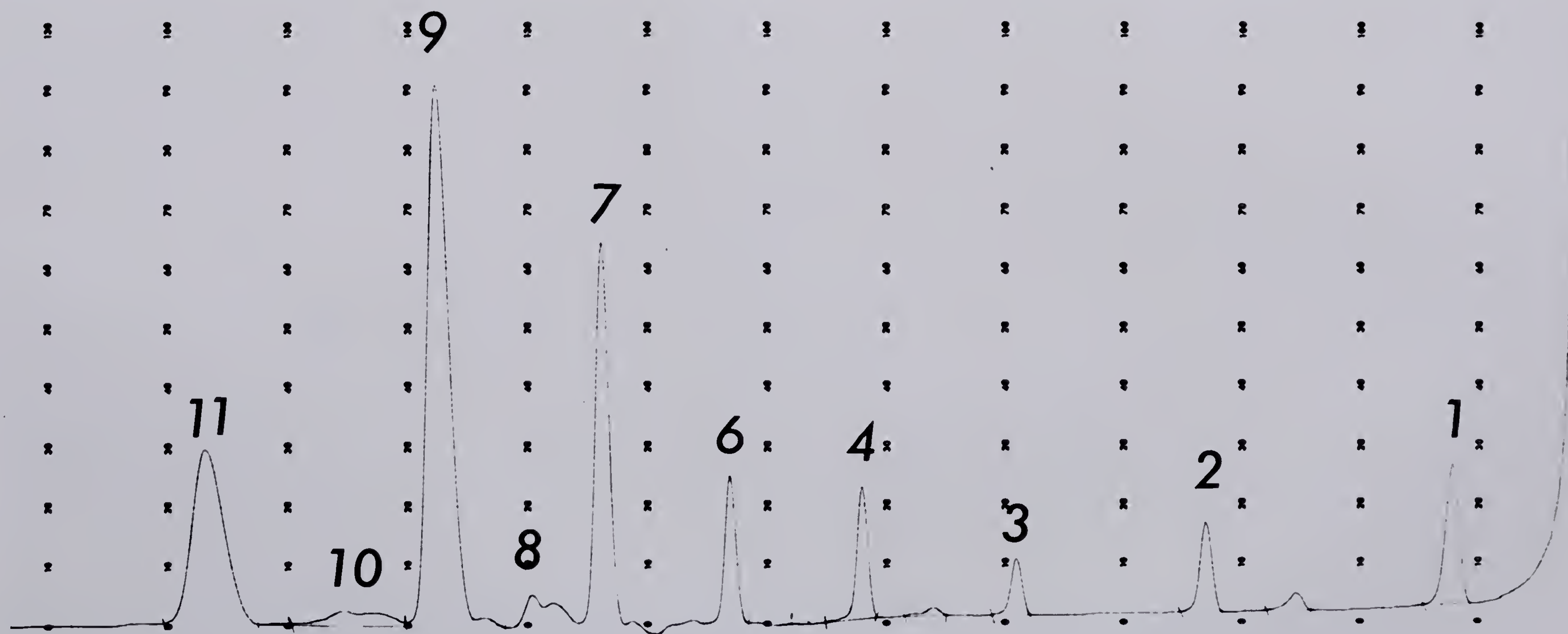

Fig. 1b. GAS CHROMATOGRAM OF METHYL ESTERS OF THE GS_3 FRACTION OF JUNE BUTTERFAT.
Peaks in order of appearance: (1) 4:0, (2) 6:0, (3) 8:0, (4) 10:0, (5) 10:1, (6) 12:0, (7) 14:0, (8) group A, (9) 16:0, (10) group B, (11) 18:0, (12) 18:1, (13) 18:2, (14) 18:3.
Peaks between (1) and (2), and (3) and (4) are not identified.

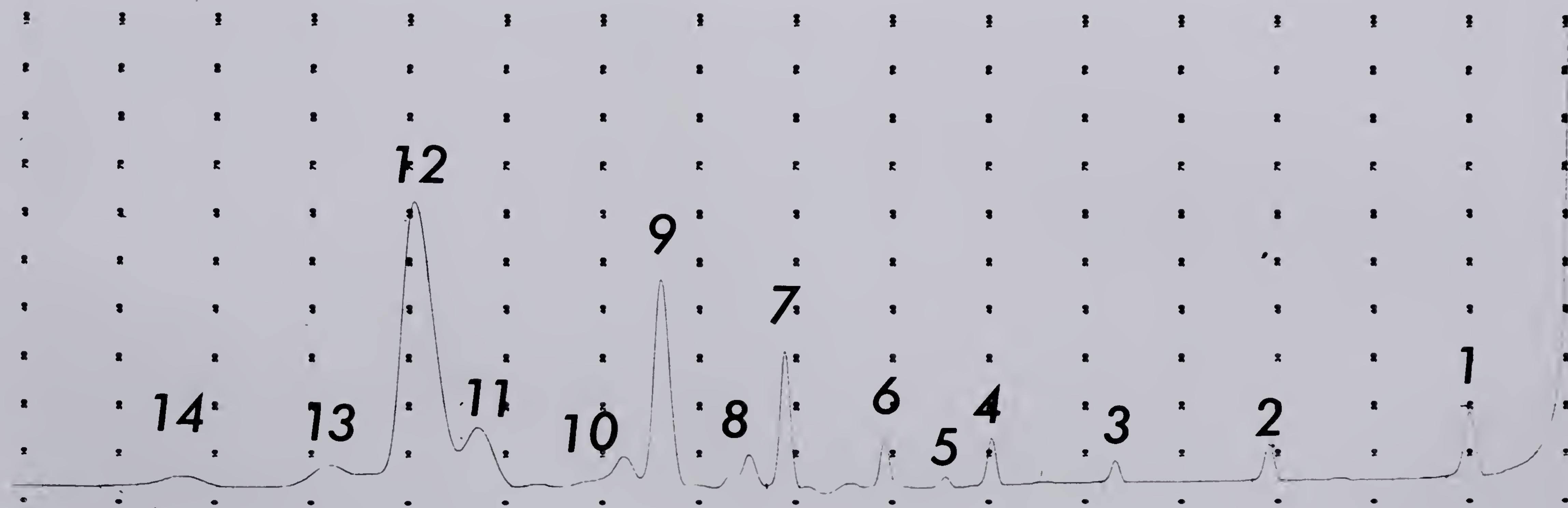

Fig. 1c. GAS CHROMATOGRAM OF METHYL ESTERS OF THE GU FRACTION OF JUNE BUTTERFAT.
Peaks in order of appearance: (1) 4:0, (2) 6:0, (3) 8:0, (4) 10:0, (5) 10:1, (6) 12:0, (7) 14:0, (8) group A, (9) 16:0, (10) group B, (11) 18:0, (12) 18:1, (13) 18:2, (14) 18:3.

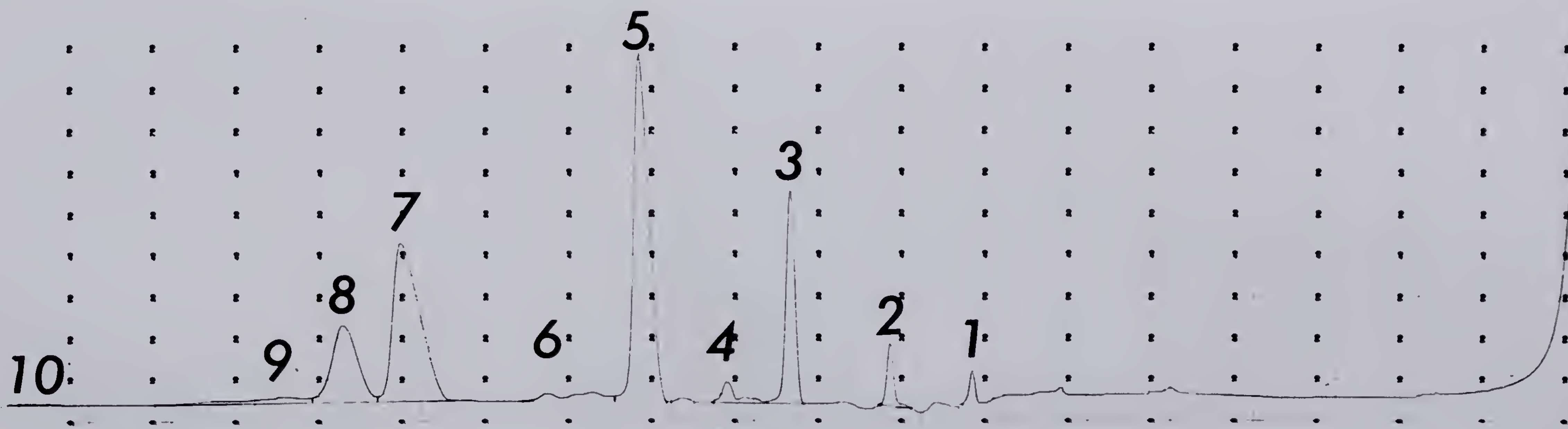

Fig. 2a. GAS CHROMATOGRAM OF METHYL ESTERS OF THE +15°C FRACTION OF JULY BUTTERFAT. Peaks in order of appearance: (1) 10:0, (2) 12:0, (3) 14:0, (4) group A, (5) 16:0, (6) group B, (7) 18:0, (8) 18:1, (9) 18:2, (10) 18:3.

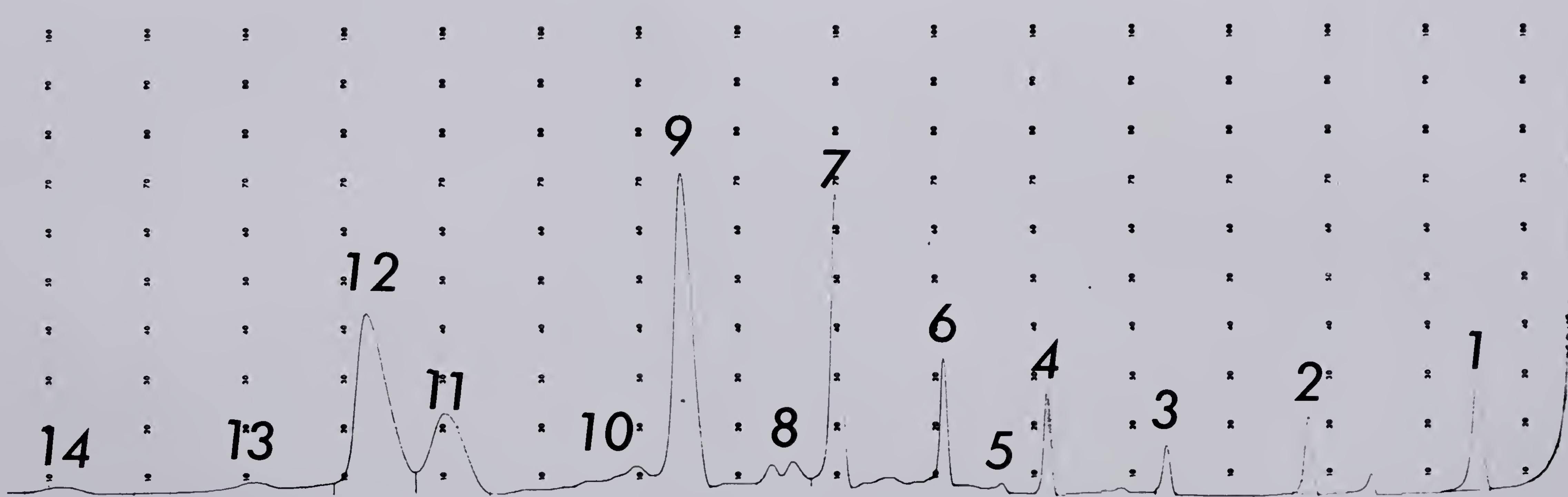

Fig. 2b. GAS CHROMATOGRAM OF METHYL ESTERS OF THE -15°C FRACTION OF JULY BUTTERFAT. Peaks in order of appearance: (1) 4:0, (2) 6:0, (3) 8:0, (4) 10:0, (5) 10:1, (6) 12:0, (7) 14:0, (8) group A, (9) 16:0, (10) group B, (11) 18:0, (12) 18:1, (13) 18:2, (14) 18:3.

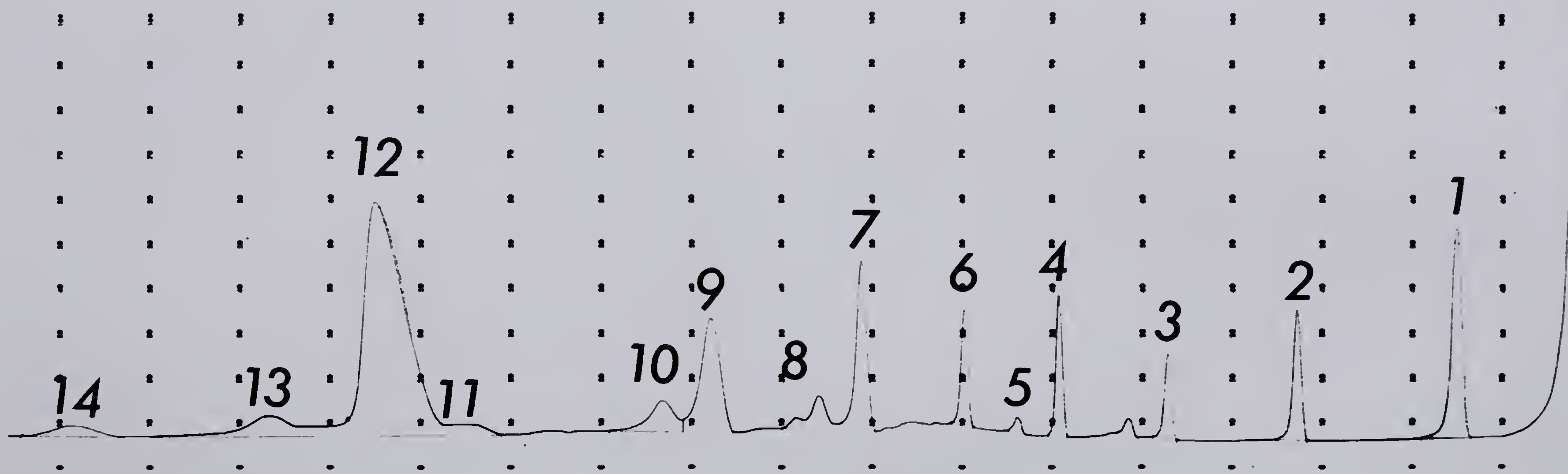

Fig. 2c. GAS CHROMATOGRAM OF METHYL ESTERS OF THE RESIDUAL FRACTION OF JULY BUTTERFAT. Peaks in order of appearance: (1) 4:0, (2) 6:0, (3) 8:0, (4) 10:0, (5) 10:1, (6) 12:0, (7) 14:0, (8) group A, (9) 16:0, (10) group B, (11) 18:0, (12) 18:1, (13) 18:2, (14) 18:3. Peak between (3) and (4) is not identified.

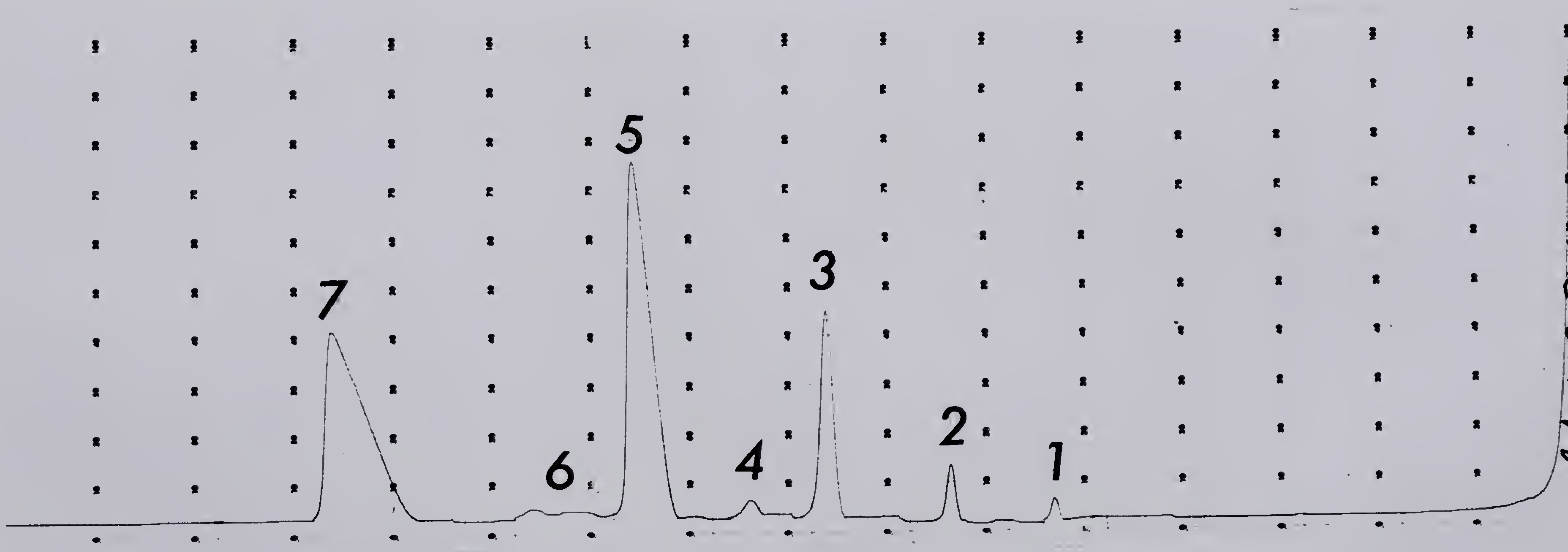

Fig. 3a. GAS CHROMATOGRAM OF METHYL ESTERS OF THE SATURATED TRIGLYCERIDES OF THE +15°C FRACTION ON JULY BUTTERFAT.
Peaks in order of appearance: (1) 10:0, (2) 12:0, (3) 14:0, (4) group A, (5) 16:0, (6) group B, (7) 18:0.

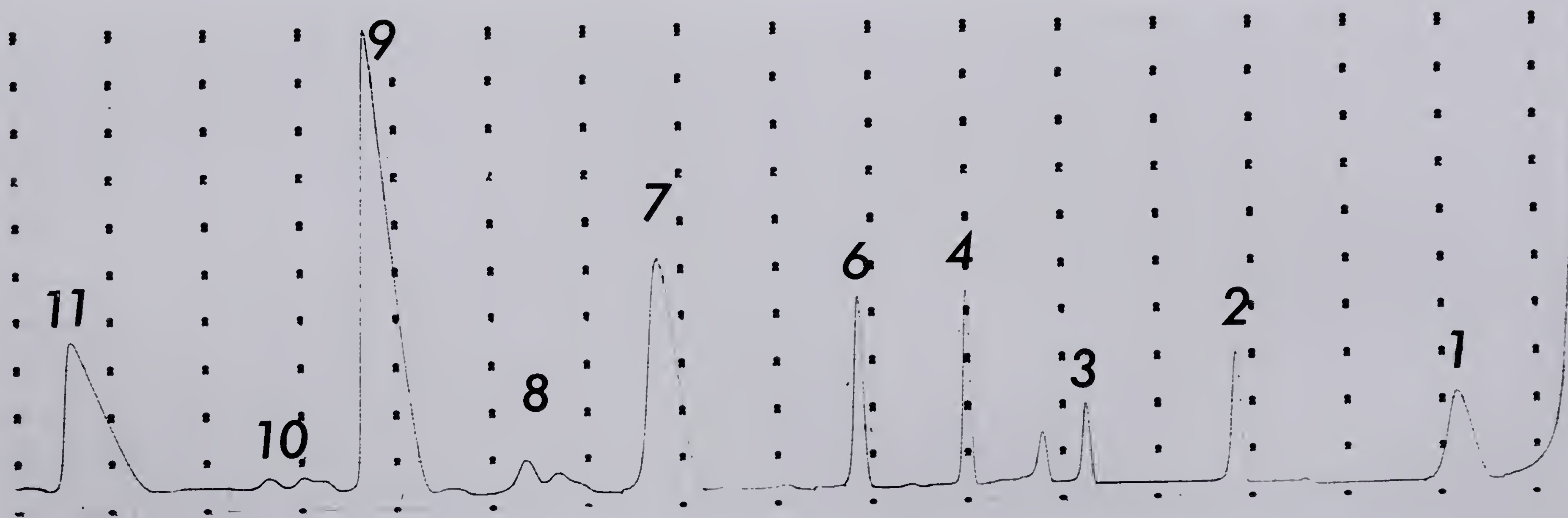

Fig. 3b. GAS CHROMATOGRAM OF METHYL ESTERS OF THE SATURATED TRIGLYCERIDES OF THE -15°C FRACTION OF JULY BUTTERFAT.
Peaks in order of appearance: (1) 4:0, (2) 6:0, (3) 8:0, (4) 10:0, (5) 10:1, (6) 12:0, (7) 14:0, (8) group A, (9) 16:0, (10) group B, (11) 18:0. Peak between (3) and (4) is not identified.

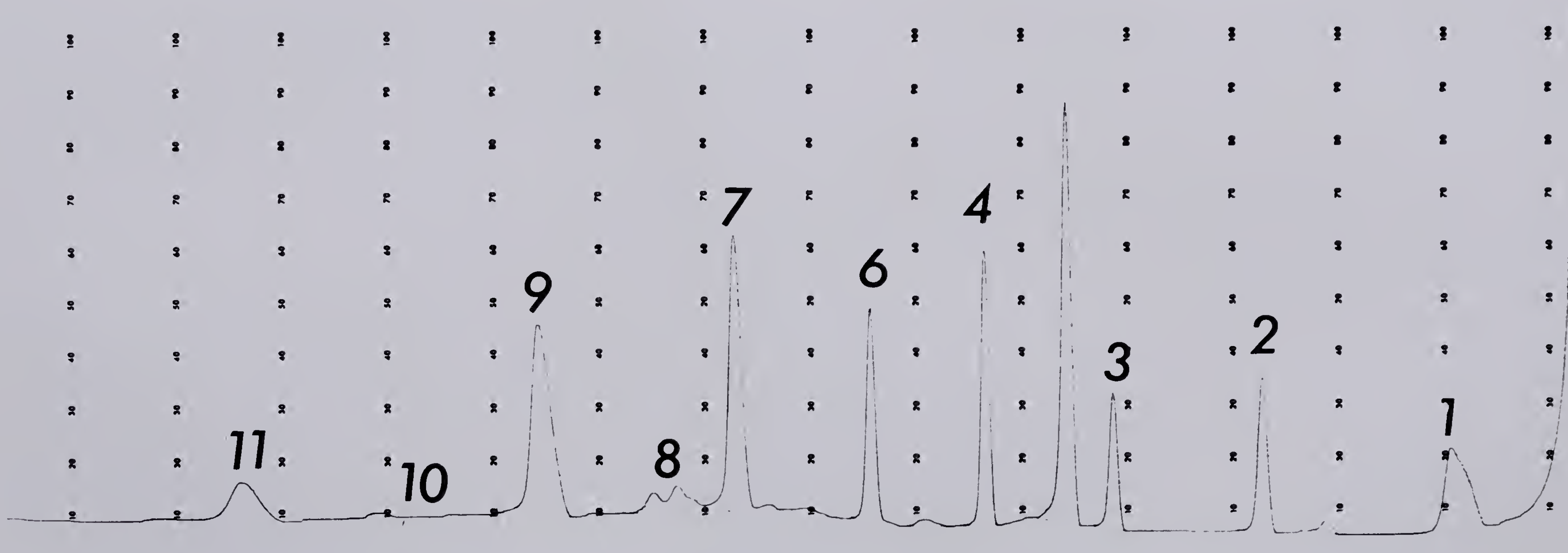

Fig. 3c. GAS CHROMATOGRAM OF METHYL ESTERS OF THE SATURATED TRIGLYCERIDES OF THE RESIDUAL FRACTION OF JULY BUTTERFAT.
Peaks in order of appearance: (1) 4:0, (2) 6:0, (3) 8:0, (4) 10:0, (5) 10:1, (6) 12:0, (7) 14:0, (8) group A, (9) 16:0, (10) group B, (11) 18:0. Peaks between (1) and (2), (3) and (4), and (4) and (6) are not identified.

DISCUSSION

(a) Procedure

The emphasis in this work was on the development of a method by which the GS_3 content could be easily determined and in which both the GS_3 and the GU fraction could be used for further analysis of the triglyceride structure. The determination of oleic acid in the fatty acid composition of the GS_3 fraction provides a reference for the completeness of the separation of the fully saturated from the unsaturated triglycerides. Oleic acid, the major unsaturated fatty acid, was only present in trace amounts in the GS_3 fractions of the butterfat samples. This absence indicates that the reaction of mercuric acetate and methanol with the double bonds of the unsaturated fatty acid residues of the triglycerides proceeded practically to completion, and that the reacted GU fraction were absorbed quantitatively by the Florisil.

The oleic acid contents of the GS_3 fractions of the samples other than butterfat indicates that the reaction did not proceed to the same extent with these fats. The reason that the reaction proceeded practically to completion with butterfat and not with the other fats might possibly be explained by steric hindrance.

Oleic acid is esterified predominantly in the external position of butterfat triglycerides (8) and in the internal position in most other natural fats (37).

It was attempted to improve the procedure for the determination of the GS_3 contents of fats other than butterfat. The addition of pyridine to the mixture of fat, mercuric acetate and methanol before

refluxing proved to be an inhibitor of adduct formation. This is in agreement with the findings of Birks and Wright (5). Borontrifluoride etherate and perchloric acid are two catalysts of which the latter proved to be more effective.

It was also investigated whether the solubility of the fat in methanol during refluxing played any role. Cacao butter, a hard and brittle fat at 5°C, did not dissolve completely during the 30 minute reflux. No consistent results were obtained when analyzing replicates of one sample of cacao butter. Butterfat dissolved completely during the 30 minute reflux, and consistent results were obtained, and oleic acid was absent in the GS_3 fractions. The addition of 50% of tributyrin to melted cacao butter made that this fat completely dissolved during refluxing, but 10% oleic acid was found in the GS_3 fraction.

The addition of chloroform to increase the solubility of cacao butter in methanol during refluxing was not effective in obtaining GS_3 free from oleic acid. Smaller sample sizes of cacao butter with the same quantities of mercuric acetate and methanol were also not effective. More research should be devoted to the GS_3 determination of fats other than butterfat in order to improve the procedure for these fats.

The advantages of the procedure are as follows:

1. The actual GS_3 fraction is separated; the determination is not based on assumptions based on poorly founded theories.

2. The GS_3 as well as the GU fractions can be used for further investigations. The method may be combined with other methods such as pancreatic lipase hydrolysis, fractional crystallization, and chromatography on silver nitrate impregnated silica gel.

3. Combination of this method with triglyceride gas-liquid chromatography would make it possible to differentiate between saturated and unsaturated triglycerides. The present triglyceride gas liquid chromatography fractionates on the basis of carbon number, independent of whether the triglycerides are saturated or unsaturated. Combination of these two methods will reveal the differences in the triglyceride distribution by carbon number between the saturated and unsaturated triglycerides.

Special interest was devoted to butterfat for several reasons. Firstly, this fat is unique among the natural fats because of its unusual fatty acid composition. Secondly, there is only very limited information on the GS_3 content and composition of this fat, because most methods will not work satisfactorily with it. Thirdly, the separation of the GS_3 is an important aid in the study of glyceride structure and synthesis of butterfat.

(b) <u>GS_3 content of natural fats</u>

1. Butterfat

Non randomized versus randomized butterfat.

As reported in Table 1 no major differences did exist between the GS_3 contents of non randomized and randomized butterfats. These same samples were used by de Man, who reported that randomization of butterfat resulted in increased softening points, hardness and high melting glycerides (14), and that randomized butterfat had greatly increased long spacing in the crystal habit (15).

Apparently randomization will cause a chance in the fatty acid distribution of butterfat triglycerides, but this will not

affect the GS_3 content or the fatty acid composition of the randomized butterfat. Hammond (27) concluded that butterfat triglycerides were arranged in a random fashion. He based his findings on the relationship between GS_3 contents, determined by the mercaptoacetic acid method, and the fatty acid compositions of the original butterfat and the trisaturated glycerides.

Kuksis, et al. (43, 44, 45) concluded that butterfat triglycerides are not arranged in a random fashion, based on triglyceride gas liquid chromatography analysis.

Butterfat samples

The GS_3 content of butterfat has in the past been determined by three methods; the permanganate oxidation method of Hilditch (13, 29, 30), the mercaptoacetic acid method (24), and the lipase hydrolysis method of Vander Wal (67). Hilditch's method causes hydrolysis of the ester groups (23, 38). The mercaptoacetic acid method has the disadvantage that the unreacted triglycerides had to be recycled twice, while an iodine value and a saponification value had to be determined on the final unreacted triglycerides in order to correct for this amount. The lipase hydrolysis method of Vander Wal (62) is based on calculations and assumptions, and the actual GS_3 fraction is not isolated. It is very likely that the action of pancreatic lipase on butterfat triglycerides follows a different pattern than it does on the triglycerides of fats other than butterfat (8). In addition, there are other possible sources of error, such as the formation of 1,3- diglycerides from 1,2-diglycerides due to isomerization (53), and the production of glycerol during hydrolysis, so that some of the free fatty acids have been split off from the 2- positions of the original triglycerides (12).

The GS_3 contents of the 19 butterfat samples listed in Table 2 ranged from 33.2% (sample 6) to 44.3% (sample 12), with an average of 39.7%. Hilditch (29) reported a range from 15 mole % to 41 mole %. Eshelman, et al. (24) reported 27.2% and 28.0%, based on the mercaptoacetic acid method. Wolf and Dugan (67) reported 34.1% in butterfat, 64.6% in the high melting glycerides of butterfat, and 71.2% in the high melting glycerides obtained from the membranes of milkfat globules. The high melting glycerides were isolated by precipitation at room temperature in 95% ethanol. The GS_3 determinations were based on the method of Vander Wal (62).

2. Fats and oils other than butterfat

The two lard GS_3 contents listed in Table 4 are 11.1% and 10.3%. These results are in close agreement with the two figures reported by Coleman (10), who obtained 9.2 mole % and 12.9 mole %. Vander Wal (62) reported 0.6 mole % and Youngs (69) 8.0 mole %. Table 9 shows that lauric acid was only found in the GS_3 fractions. The probable reason is that lauric acid is more concentrated in the GS_3 fractions and can thus be detected. Magidman, et al. (48) reported 0.07% lauric acid in lard.

It is remarkable that the stearic acid content of the GS_3 is about four times higher than in the whole fat, whereas the palmitic acid content is about double of that in the original fat. It is likely that proportionally more stearic acid is combined with the saturated triglycerides than is palmitic acid. Jurriens and Kroesen (37) obtained similar results for the fatty acid compositions of the whole fat and that of the "0 double bond" fraction. They reported 6.6 mole % for the "0 double bond" fraction calcualted from the fatty acid composition of this fraction.

The two shortening samples listed in Table 4 contained 17.1% and 11.5% GS_3. Probably these results are the first to be reported on shortening samples. Sample 1 has a higher percentage of saturated fatty acids (Table 10) and a correspondingly higher GS_3 content. More stearic than palmitic acid was combined with the saturated triglycerides, especially in sample 2.

The GS_3 contents in the margarine fat samples ranged from 4.1% to 9.3% (Table 4). As was the case in the shortening samples the fat with the highest saturated fatty acid content also had the highest GS_3 content. Samples 3 and 5 contained marine oils, the other samples contained only vegetable oils. Samples 3 and 5 had approximately the same GS_3 contents (9.0 and 9.3%).

Coconut oil had a GS_3 content of 81.9%. Eshelman, *et al.* (24) reported 72.2 and 76.2% by the mercaptoacetic acid method. Collin and Hilditch (13) obtained 84 - 86 mole %. It is interesting to note that the content of palmitic acid in this case was higher in the GU fraction than in the GS_3 fraction.

(c) Butterfat fractions obtained by fractional crystallization from acetone

Table 3 gives the GS_3 contents of the fractions obtained by fractional crystallization from acetone. The +5°C fraction has a higher GS_3 content than the +15°C fraction, although it is to be expected that the greater amount of oleoglycerides would result in a lower GS_3 content in the +5°C fraction. Table 13 shows the absence of short chain fatty acids in the +15°C fraction, they are present in all of the lower melting fractions. The occurrence of triglycerides which have a short chain fatty acid or oleic acid in the +5°C fraction is to be a certain extent at the

expense of saturated triglycerides with long chain fatty acids. Since the GS_3 content is higher in the +5°C fraction than in the +15°C fraction we can deduce that with decreasing melting point of the two fractions there is a greater increase of saturated triglycerides with a short chain fatty acid than there is an increase in the amount of oleoglycerides.

We may expect that the contents of stearic and palmitic acid would decrease in successively lower melting fractions. However, only the content of stearic acid decreases with decreasing melting points. Palmitic acid is relatively constant in concentration in the +15°C-, +5°C-, and -5°C fractions and also in the saturated glycerides of these fractions and those of the -15°C fraction.

These considerations lead to the conclusion that butyric acid in the high melting fractions is most likely mainly combined with palmitic acid in the glycerides. Further study, especially by GLC analysis should verify this point.

The residual fraction of July butterfat has a surprisingly high GS_3 content (27.3%) but the butyric acid content of the GS_3 is relatively low. The butyric acid content in the GU fraction calculated by difference is:

$$100 \times 7.3\% = 27.3 \times 7.1 + 72.7 \times \text{butyric acid \% in GU},$$

or: butyric acid in GU = 7.4%

and $$\frac{72.7 \times 7.4}{730} \times 100 = 74\% \text{ of the butyric acid is combined}$$

with unsaturated fatty acids, of which oleic acid is the major one, to form the GU fraction.

(d) Fatty acid composition of butterfat and the various fractions obtained from these samples

The fatty acid compositions listed in Tables 5, 6 and 7 indicate that there are only minor differences between the fatty acid compositions of the GS_3 and GU fractions of non randomized and randomized butterfat samples. With the exception of sample 9 in Table 8 the fatty acid compositions of the GS_3 fractions of these samples appear to follow a random pattern. These results agree with the findings of Hammond (27). In the fractions, obtained by fractional crystallization from acetone, butyric acid seems to be combined with palmitic acid in the high melting glyceride fractions and with oleic acid in the low melting glyceride fractions. The overall appearance of a butterfat fatty acid composition in relation to those of the GS_3 and GU fractions is random, but if this butterfat is fractionated it looses its random appearance.

This conclusion is supported by the work of Dimick, et al., (20) who reported that palmitic acid is randomly distributed on the glycerol molecules of butterfat triglycrides, but it looses this appearance if these glycerides were fractionated according to molecular weight by silica gel column chromatography. In the high molecular weight triglycerides palmitic acid was esterified on the internal position, and in the low molecular weight triglycerides on the external positions.

(e) Unusual peaks in gas chromatograms of the GS_3 and GU butterfat fractions

In the gas chromatograms of these samples peaks representing unknown compounds were noticed mostly in the GS_3 fractions. The peaks had retention volumes between butyric and caproic acid (designated "a" in the fatty acid composition tables), between caprylic and capric acid ("b"), and in the low melting GS_3 fractions between capric and

lauric acid ("c"). These peaks were also noticed in the original samples if the chromatograms were run with a higher sensitivity between the major peaks, and in the July butterfat low melting fractions without higher sensitivity. The amount of 16.8% w/w for "b" in the GS_3 residue fraction cannot be accounted for. Zeman and Pokorny (63) identified heptanoic, nonanoic, undecanoic acid and its branched isomer. Nonanoic acid might be the compound "b", and undecanoic acid might be the compound "c".

A chromatogram of completely hydrogenated butterfat had peaks corresponding to "a", "b" and "c" with the same retention volumes, indicating that these peaks most likely represent saturated fatty acid esters. Some minor peaks in the GU fractions of some samples did also occur. It is possible that these peaks represent hitherto unknown and unidentified minor fatty acids or they might be impurities or artefects.

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